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The toxic effects of microcystin-LR on mouse lungs and alveolar type II epithelial cells

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Abstract

Objectives—Microcystin-leucine arginine (MC-LR) is produced by cyanobacteria and can accumulate in lungs through blood circulation. However, the effect of MC-LR on lung remains unclear. In this study, we investigated the chronic, low-dose effect of MC-LR on mouse lung tissues and the influence of MC-LR on mouse alveolar type II epithelial cells (ATII cells).

Methods—MC-LR was orally administered to mice at 0, 1, 10, and 40 µg/L for 6 consecutive months and mouse lungs were obtained for histopathological and immunoblot analysis. ATII cells were cultured in various concentrations of MC-LR (0, 0.5, 5, 50, 500 nmol/L) for indicated time and the cell viability and proteins change were tested.

Results—Our study revealed that the chronic, low-dose MC-LR exposure induced alveolar collapse and lung cell apoptosis as well as the breach of cell junction integrity. Furthermore, following treatment with MC-LR, ATII cells could uptake MC-LR, resulting in apoptosis and disruption of cell junction integrity.

Conclusions—These data support the toxic potential of low-dose MC-LR in rendering chronic injury to lung tissues.

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Ethics statement: The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006) and all experimental protocols were approved under the animal protocol number SYXK (Su) 2009-0017 by the Animal Care and Use Committee of Nanjing University.

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Keywords

Microcystin-LR; Chronic low dose; Lung; Toxic

1. Introduction

Microcystins (MCs) are a class of naturally produced cyclic heptapeptide toxins produced by cyanobacteria in freshwater (Carmichael et al., 2001). According to their amino structure, MC analogs can be divided into MC-leucine arginine (MC-LR), MC-arginine arginine (MC-RR) and MC-tyrosine arginine (MC-YR). MC-LR is the most abundant and the most toxic MC variant in mice (Gupta et al., 2003), and the provisional guideline set by the World Health Organization (WHO) for microcystin-LR (MC-LR) in drinking water is 1 µg/L (Dietrich and Hoeger, 2005). However, the concentrations of MCs in many water bodies are far beyond that guideline. One study showed that the level in the Poyang Lake, the largest freshwater lake in China, was 7.97 µg/L (Zhang et al., 2015), whereas in the Hoan Kiem Lake of northern Vietnam, a concentration of MCs as high as 46 µg/L was recorded (Duong et al., 2013). MC-LR is chemically stable and has a very slow natural degradation process. Consumption of the fish that living in the water contains MCs may pose potential risks to the health of humans (Jia et al., 2014). MCs have been shown the toxic effects to liver, nerve, kidney and reproductive organs (Funari and Testai, 2008).

MC can be transported via the bloodstream rapidly and distributed to various organs once absorbed into the blood. Wang et al. and his colleagues studied the distribution of MCs in various tissues of Wistar rats under laboratory conditions, and they observed the highest concentration of MCs in the lung after kidney (Wang et al., 2008). Furthermore, it is demonstrated that MCs are toxic to the lung and found that MCs can induce apoptosis and inflammation in lung tissues, as well as a rapid increase in lung impedance (Soares et al., 2007).

Wang and Takano both found that MC-LR can influence the expression of tight junctions, which is required for embryonic development and cancer metastasis (Wang et al., 2005), as well as the gas exchange of the lung (Overgaard et al., 2012). The lung must maintain a highly specialized barrier between the atmosphere and fluid-filled tissues to initiate gas exchange. Recently, it is increasingly recognized that the epithelial mesenchymal transition (EMT), which is also involved in cancer invasion and metastasis as well as embryonic development (Ahmed et al., 2006), can affect the lung respiratory function. When triggered by local factors such as transforming growth factor- β (TGF- β), alveolar epithelial cells may differentiate into mesenchymal cells after losing the tight junctions and epithelial cell markers. However, in the previous studies, which were designed to investigate the acute or subacute toxic effects of MC-LR, the animals received high doses of MC-LR in a short time period. The mode of delivery, by intraperitoneal or intravenous injection with relatively high doses of MC-LR, does not represent typical ways by which the organisms may come into contact with this toxicant. To mimic a more natural exposure condition, the present study has applied the lifetime safe consumption level proposed by the WHO for MC-LR (1 µg/L) as the basic treatment dose and 40 µg/L as the highest dose. Based on the general amount of

water intake by mice, the concentrations of 1, 10 and 40 µg/L MC-LR are calculated to correspond to about 0.1, 1, 4 µg/kg bw day, respectively. MC-LR was dissolved in the daily drinking water of mice to study the toxic effects of chronic, low-dose exposure to MC-LR on the lungs.

In addition, the mechanism by which MC-LR causes lung injury is not clear. Therefore, we explored the influence of MC-LR on alveolar type II epithelial cells (ATII cells), which form the most important bioactive component of pulmonary epithelium and secrete pulmonary surfactants (Rooney et al., 1994). Moreover, ATII cells are interspersed throughout the alveoli to form tight junctions (Hermanns et al., 2009). It is well known that MC-LR is a potent inhibitor of serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A), which can have a strong impact on the activity of phosphoproteins. As direct or indirect substrates of PP2A, several protein kinases have been reported to be phosphorylated dose-dependently after exposure to MC-LR, which include the phosphatidylinositol 3-kinase (PI3-K)/Akt and mitogen/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways. The activation of the PI3K/Akt and MEK/ERK signaling pathways have been thought to promote proliferation, differentiation, and survival. Recent findings implicated these pathways in apoptosis induction in several cell types under genotoxic or nongenotoxic stresses (Cagnol and Chambard, 2010). However, the functions of ERK on MC-LR exposure are obscure and even controversial (Dias et al., 2010). In the present work, the involvement of PI3K/Akt and MEK/ERK signaling pathways in this regard was explored.

In this study, we first investigated the effect of MC-LR on ATII cells *in vitro* and the impact of chronic, low-dose exposure to MC-LR on the lungs *in vivo*, which has helped us to gain insight into the mechanisms of toxicity associated with chronic, low dose MC-LR exposure to the respiratory system.

2. Materials and methods

2.1. Animals and treatment

The experiment was performed as previously described (Chen et al., 2011). Briefly, specific pathogen free (SPF) female BALB/C mice (6–7 week old) (n = 40) weighing from 20 to 25 g were purchased from Experimental Animal Center of the Academy of Military Medical Science Institute, China. After 1 week of acclimation, mice were randomly divided into 4 groups: one control and three experimental with 10 mice in each group. MC-LR was purchased from Alexis Biochemicals (Switzerland). The range of the MC-LR doses was chosen from between 1 µg/L, the recommended upper level by WHO for drinking water, and 40 µg/L. In the experimental groups, mice were exposed orally at 1 µg/L, 10 µg/L, and 40 µg/L MC-LR for 6 consecutive months. Mice were sacrificed later and mouse lungs were obtained for histopathological analysis.

2.2. Histology

The experiment was performed as previously described (Wang et al., 2015). Briefly, lower left lungs were cut into 5-µm paraffin sections followed by staining with hematoxylin-eosin

(H&E) for structural observation, TdT-mediated dUTP nick end labeling (TUNEL) for apoptosis, and immunofluorescence for protein regulation. Alveolar collapse is defined as the loss of normal alveolar structure.

2.3. Immunofluorescent staining

Immunofluorescence analysis was performed as described previously (Wang et al., 2014). The following primary antibodies were employed: Rabbit anti-zonula occludens-1 (ZO-1); rabbit anti-occludin (all antibodies were purchased from Abcam, Cambridge, MA). Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen) was used as the secondary antibody. The images were captured using a confocal fluorescence microscope (Olympus, Tokyo, Japan).

2.4. TUNEL staining

TUNEL was performed as described previously (Chen et al., 2011). Briefly, 5- μ m sections of lung tissues were labeled DNA strand breaks with fluorescein-12-dUTP, the sections were mounted in mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) for nuclear visualization, and images were examined by a confocal fluorescence microscope (Olympus, Tokyo, Japan) to detect localized green fluorescence from apoptotic cells.

2.5. Myeloperoxidase (MPO) activity assay

The lung tissue was lysed with myeloperoxidase (Campos and Vasconcelos) assay buffer and the MPO activity was detected using a kit from Nanjing JianCheng Bioengineering Institute (Nanjing, China) according to the instruction by the manufacturer.

2.6. ATII cell culture

The mouse ATII cell line was purchased from Yili Bio-technology (Shanghai, China). The cell line exhibits characteristics of ATII cells such as lamellar bodies (Ultrastructural features of ATII cells under electron microscopy) and expression of cytokeratin (CK) 18, CK19, occludin and surfactant protein C (SP-C); the cells were immortalized by SV40 transfection. ATII cells were cultured in high DMEM (Hyclone) with 10% FBS (GIBCO), 1% L-glutamine, and a 1% solution of penicillin and streptomycin. Cells were routinely passaged using 0.25% trypsin when they reached 80% confluence, and were diluted 1:3 at each passage.

2.7. CCK-8 assay

Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) is based on the conversion of an orange-colored product from water-soluble tetrazolium salt by dehydrogenases in live cells. ATII cells were cultured in various concentrations of MC-LR (0, 0.5, 5, 50, 500 nmol/L) for 12 h in 96-well plates followed by the CCK-8 assay according to the instruction from the manufacturer.

2.8. FDA and PI staining for morphologic evaluation

The fluorescein diacetate (FDA)/propidium iodide (PI) staining was performed as previously described (Wang et al., 2015). Cells were placed on a 24-well culture plate at 1×10^6 cells/ml in 0.5 ml culture medium and cultured for 12 h followed by treatment with various

concentrations of MC-LR as indicated for 12 h. Next, the cells were stained with 5 µg/ml PI and 4 µg/ml FDA, followed by observation under a fluorescent microscope (Nikon ECLIPSE TE2000-S, Tokyo, Japan).

2.9. RNA extraction and quantitative real-time polymerase chainreaction (Q-PCR)

The RNA extraction was performed as previously described (Wang et al., 2015). The sequences of primer pairs used in this assay are shown in Table 1. Q-PCR was conducted by amplifying 20 µl of diluted cDNA with the SYBR Green Q-PCR kit (Roche, Germany) on a 7300 Real-Time PCR System (ABI, America).

2.10. Western blotting

Proteins were purified from lungs or ATII cells after exposure to MC-LR for 48 h. Western blot analysis of cellular lysates was performed as previously described (Wang et al., 2014). The following primary antibodies were employed: Rabbit anti-CK18, rabbit anti-CK19, rabbit anti-SP-C, rabbit anti-E-cadherin, rabbit anti-ZO-1, rabbit anti-occludin, rabbit anti-vimentin, rabbit anti-ERK1/2, rabbit anti-p-ERK1/2, rabbit anti-Akt, rabbit anti-p-Akt, mouse anti-MEK1/2, rabbit anti-p-MEK1/2, rabbit anti-PTEN, rabbit anti-GAPDH, mouse anti-MEK1/2 and mouse anti-β-actin (Abcam Inc. Cambridge, MA). Horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (Boster, Wuhan, China) was used as the secondary antibody. Immunoreactive protein bands were detected using an Odyssey Scanning System (LI-COR Inc).

2.11. Transepithelial electrical resistance (TEER)

ATII cells were grown on polycarbonate transwell inserts with a 0.4-µm pore size for 12 well multiwell tissue culture plates (Corning, Lowell, MA). After cells reached confluence, various concentrations of MC-LR were added to the medium. Next, the TEER of epithelial monolayers was measured using Millicell-ERS (Electrical Resistance System) from Millipore at 12, 24 and 36 h. Six independent experiments each on a separate filter were performed.

2.12. Statistical analysis

Experimental results were expressed as mean ± standard deviation. Differences were analyzed for significance ($P < 0.05$) by one-way ANOVA using SPSS for windows version 11.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. MC-LR induces the alveolar septa thickening based onhistopathological evaluation

Although the presence of MC-LR was not observed by immunoblot analysis in the mouse lungs after exposure to MC-LR for 6 months (data not shown), we explored the structural changes of the mouse lungs of the exposed mice. With the increased concentration of MC-LR, an increase in collapsed areas and thickening of the alveolar septa can be observed. Alveolar walls appeared different degree of fracture and some parts of the fractured alveolar fused. Treatment with 40 µg/L MC-LR induced the most obvious alveolar collapse in

contrast to a regular arrangement of alveolar structure in control tissues, and tissues from the mice treated with 1 µg/L and 10 µg/L (Fig. 1 A).

3.2. MC-LR induces cell apoptosis without detectable inflammation in mouse lung tissues

In comparison with the control group, no significant difference in the number of apoptotic cells at MC-LR doses of 1 and 10 µg/L was observed. However, at 40 µg/L the numbers of apoptotic cells increased slightly (Fig. 1B). MPO is the characteristic of the neutrophils, and the MPO activity can reflect the amount of infiltrating neutrophils that is one of the hallmarks of inflammation (Campos and Vasconcelos). In order to test the level of inflammation induced by MC-LR, we measured the quantity of MPO and the expression of a group of common inflammatory factors (i.e., IL-1β, IL-6 and TNF-α). No MC-LR-mediated inflammation could be observed at all the concentrations tested (Fig. 1C, D).

3.3. MC-LR up-regulates EMT-related molecules in mouse lungs

We investigated the expression of the levels of EMT-related proteins including the cell adhesion protein, the epithelial protein, and the mesenchymal protein in lung tissues following oral administration of MC-LR for 6 months. The levels of E-cadherin, SP-C, occludin and ZO-1 were significantly down-regulated compared with control. Moreover, the expression of TGF-β and vimentin was significantly up-regulated at MC-LR doses of 10 µg/L and 40 µg/L compared with control (Fig. 2).

3.4. MC-LR can be uptaken by ATII cells resulting in viability loss of ATII cells

ATII cells were incubated in various concentrations of MC-LR for 12 h followed by western blotting for the detection of MC-LR. MC-LR was found to be associated with the cells treated with MC-LR at 50 and 500 nM (Fig. 3A). Next, the intracellular presence of MC-LR in ATII cells was confirmed by immunofluorescent staining using confocal microscopy after the cells were incubated at 500 nM of MC-LR for 12 h (Fig. 3B). The viability of ATII cells exposed to MC-LR for 12 h was assessed by the CCK-8 assay. Cell viability was significantly decreased following exposure to 50 and 500 nM MC-LR. No significant differences in cell viability were observed between groups exposed to a concentration not higher than 5 nM (Fig. 3C). Apoptosis of ATII cells exposed to different concentrations of MC-LR was measured with FDA and PI staining. We demonstrated that MC-LR, at a concentration higher than 50 nM, could significantly increase the death of ATII cells. No significant differences were observed between control and exposure at concentrations of 0.5 and 5 nM (Fig. 3D).

3.5. MC-LR promotes EMT in ATII cells

We investigated the expression levels of EMT-related proteins by western blotting and immunofluorescence assay. Therefore, epithelial markers, cell adhesion proteins, tight junction proteins and mesenchymal proteins in ATII cells following exposure to various concentrations of MC-LR for 48 h were measured. With increasing concentrations of MC-LR, the expression levels of CK18, CK19, SP-C, occludin, E-cadherin and ZO-1 were significantly down-regulated. In contrast, the expression level of vimentin was significantly up-regulated (Fig. 4).

3.6. MC-LR exposure results in a rapid decrease in TEER in a dose-dependent manner

Disruption of epithelial barrier function can be measured by changes in TEER. Confluent monolayers of the transformed ATII cells were treated with increasing concentrations of MC-LR, ranging from 0 to 500 nM. Measurements of TEER were performed after 12, 24, and 36 h. As shown in Fig. 5 A, the TEER decreased in a dose-dependent manner with increasing concentrations of MC-LR.

3.7. MC-LR activates PI3-K/AKT and MEK/ERK in ATII cells

Next, we investigated the effect of MC-LR on the expression of PI3-K/AKT and MEK/ERK at the protein level. ATII cells were treated with various concentrations of MC-LR (0, 0.5, 5, 50, or 500 nM) for 12 h followed by western blotting. MC-LR exposure at 5, 50 and 500 nM induced phosphorylation of AKT, MEK and ERK. MC-LR decreased the PTEN protein expression in a dose dependent manner (Fig. 5 B). These results indicated that PI3-K/AKT and MEK/ERK could be activated by MC-LR in ATII cells.

4. Discussion

As an important environmental pollutant, MC-LR is the most abundant and the most toxic MC (Gupta et al., 2003). MC-LR entering via an oral route could cause histological damages to the stomach and small intestine, and it can enter the blood via capillaries in the intestinal villi (Ito et al., 2001). There are several authors who have clearly and earlier demonstrated nephrotoxicity and hepatotoxicity, some of them on human cells (Milutinovic et al., 2003; Sun et al., 2014). We have also demonstrated that chronic, low dose treatment with MC-LR may result in substantial toxicity to male reproduction, causing injury to the testis and declines in sperm quality (Chen et al., 2011). However, accumulating evidence has indicated that the lung might be another important target organ of MC and MC-LR may exert both acute and subacute toxic effects on the lung via intraperitoneal or intravenous injection (Ito et al., 2001). Nevertheless, the mode of MC-LR delivery in these studies does not represent natural exposure.

In the present study we investigated the effect of chronic, low-dose exposure to MC-LR via the oral route. We failed to detect the presence of MC-LR in lung tissues by immunoblot analysis. In the research of Wang et al. (2008), rats were administered by an i.v. injection of MCs at a dose of 80 mg MC-LR equivalent/kg body weight and the quantitative analysis of MCs was determined by Liquid Chromatography-Mass Spectrometry (LC-MS). However, in our present study, we use a much lower concentration (as high as 4 µg/kg bw day in drinking water) of MC-LR and we detected the presence of MC-LR in lungs by immunoblot analysis. Due to the differences in experimental method, we failed to detect the presence of MC-LR. Another possible explanation for this maybe that the concentration of MC-LR in the lung was below the detection sensitivity, which highlights the substantial toxic potential of MC-LR in the tissues. Of course it cannot be ruled out that MC-LR could have been metabolized in the lungs to other forms which could not be recognized by the antibodies. Despite no obvious MC-LR deposit detected in the lung, the alveolar collapse and cell apoptosis appeared when mice ingested MC-LR (40 µg/L) for 6 months in drinking water. Alveolar septa thickening and alveolar collapse is the most directly evidence indicating that

MC-LR was toxic to lung. The concentration of MC-LR is the only variable between each group, this is sufficient to link the observed pathology to the direct effect of MC-LR. In this study, dose-dependently increased alveolar septa thickening and alveolar collapse following exposure to MC-LR was observed, proving that MC-LR could change the structure of lung and maybe affect the function of respiratory system. In addition, we found that MC-LR can induce cell apoptosis in the lung, as identified in previous study (Mouded et al., 2009). X. Li et al. have shown HE staining of lungs exposed to MC-LR for 12 months (Li et al., 2015), the different experimental period and the different source of animals may be the reasons for quite different pathology. MC-LR is able to disturb the immune system (Yuan et al., 2012). Experimental animals treated with MCs may be less resistant to infections after long time exposure to MC-LR. This may be another important factor that induce differences between our present study and the results in the recently published article by X. Li and we will do the research in the future.

Many acute studies showed that MCs can induce inflammation in the lung (Casquilho et al., 2011). Therefore, we investigated the expression levels of some most common inflammatory cytokines, including IL-1 β , IL-6 and TNF- α , as well as neutrophil infiltration. However, we failed to observe signs of inflammation in the lung tissues, which suggested that chronic, low-dose exposure to MCs may not induce obvious inflammation.

One of the consequences of the alveolar epithelial cell injury as a result of lung invasion of adverse factors is the production of TGF- β which can promote the alveolar epithelial cells to differentiate into mesenchymal cells after losing the epithelial cell markers and cell junctions. Cell-to-cell communication is very important to maintain the normal function of the organs. EMT may compromise cell-to-cell communication which is characterized by the up-regulation of vimentin and down-regulation of E-cadherin (Tsukamoto et al., 2013). In this study, the expression levels of E-cadherin, occludin and ZO-1 were significantly down-regulated, whereas the levels of TGF- β and vimentin were up-regulated at MC-LR doses of 10 and 40 μ g/L. Taken together, these data suggested that MC-LR may damage the cell-to-cell communication, which is critical for maintaining the normal function of the lung.

There are several types of intercellular junctions between alveolar epithelial cells. Of these, tight junction is the most critical for the epithelial barrier function (Komarova et al., 2007). Since not all MC-LR can accumulate in the lung, in the present study, ATII cells were treated with a relative low dose of MC-LR and the presence of MC-LR was detected by immunoblot analysis. In *in vivo* study, MC-LR was absorbed from digestive tract, then spread all over the body after the metabolism of the liver. The concentration of MC-LR may be decreased sharply, or MC-LR could have been metabolized in the liver or lung to other forms which could not be recognized by the antibodies. However, in *in vitro* study, MC-LR was directly added into the cell culture medium and can be absorbed effectively. Therefore, it is reasonable to detect the presence of MC-LR in alveolar epithelial cells in culture and the data of *in vivo* study fit to the data of *in vitro* study.

ATII cells are interspersed throughout the alveoli and serve important functions including synthesis and secretion of pulmonary surfactants (Wu et al., 2015). SP-C is one of the important pulmonary surfactants that can reduce the surface tension at the alveolar air liquid

interface and provide alveolar stability necessary for normal ventilation (Mason et al., 2000). We found that the expression of SP-C was decreased in both mouse lungs and ATII cells, confirming the damage to the normal function of the lung, after exposure to MC-LR. In addition, up-regulation of vimentin suggested the differentiation of ATII cells into mesenchymal cells. Permeability barrier function is established by tight junctions and TEER is increased with the formation of tight junctions (Buchert et al., 2012). In our present study, the TEER in ATII cells was extremely down-regulated after exposure to MC-LR. Taken together, these data support the adverse effect of MC-LR on the disruption of tight junctions and induction of EMT.

MC-LR could up-regulate EMT-related molecules and damage tight junction both in *in vivo* and *in vitro* study. Because we detected the presence of MC-LR in alveolar epithelial cells in culture instead of lung tissues, we did more research in *in vitro* study. MC-LR is a potent inhibitor of serine/threonine protein PP1 and PP2A (Campos and Vasconcelos, 2010), which can have a strong impact on the activity of phosphoproteins, DNA repair systems and gene expression. The activation of the PI3K/Akt and MEK/ERK signaling pathways have been suggested to be involved in the apoptosis induced by MC-LR (Wang et al., 2012). Moreover, MC-LR exposure could increase melanoma cell invasion and tumor metastasis as well as EMT by activating PI3K/AKT (Tsukamoto et al., 2013; Xu et al., 2013). In addition, it is possible that the activated MAPK signaling pathways play important roles in modulating MC-LR-induced cellular stress responses (Carreras and Poderoso, 2007). In our present study, we failed to detect the change of protein PP1 and PP2A activity in lung tissues by immunoblot analysis. However, in *in vitro* study, the phosphorylation of these downstream proteins was markedly up-regulated after MC-LR treatment in a concentration-dependent manner. Thus, MAPK and AKT might be involved in mediating the responses of ATII cells to MC-LR exposure through the regulation of the phosphorylation of these proteins, which was negatively correlated with PP2A activity.

Despite the overwhelming pathologies observed in lung tissues after oral exposure of the mice to MC-LR, we did not detect any tumors or fibrosis in mouse lungs. However, it has been consistently reported that the occurrence of EMT can be regarded as the initiator of these pathologies (Milutinovic et al., 2006; Zubeldia et al., 2013). Furthermore, loss of cell junctions can lead to cancer development (Wang et al., 2005). Epidemiological studies have demonstrated that MCs may contribute to a higher incidence of primary human carcinomas (Zhou et al., 2002). Experimental evidence also supports that MC-LR may not only act as a tumor initiator, but also stimulate the invasion and migration of cancer cells (Xu et al., 2013; Zhang et al., 2012). It is also documented that MC-LR can promote fibrosis (Milutinovic et al., 2006). Therefore, we may predict that animals or humans, if routinely ingest MC-LR-contaminated foods or liquids, may develop chronic lung injury, resulting in lung cancer and fibrosis.

In conclusion, our present results demonstrated that chronic, low-dose exposure to MC-LR was toxic to the lung and could compromise cell-to-cell communication by decreasing the integrity of intercellular junctions. This study may increase our understanding of the toxic effects of MC-LR on the respiratory system.

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Fig 1.

Mice were administered with MC-LR at 1 µg/L, 10 µg/L, and 40 µg/L in drinking water for 6 consecutive months. Mice were sacrificed and lungs were obtained. (A) Lung tissues were stained with H&E (200×). With the increased concentration of MC-LR, an increase in collapsed areas and thickening of the alveolar septa can be observed (arrow). (B) Fluorescent images of lung tissues following double staining for TUNEL (yellow) and DAPI (blue) (600 ×) are shown (left panel). The percentages of TUNEL positive cells were quantified (right panel) (n = 5 per group). (C) Myeloperoxidase activity was measured to reflect the amount of neutrophils (n = 5 per group). (D) RNA was isolated, and expression of IL-1β, IL-6 and TNF-α were measured by real-time quantitative polymerase chain reaction. Results were normalized to GAPDH (n = 5 per group). Data are shown as mean ± SD. **P* < 0.05 vs. control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig 2.

Mice were administered with MC-LR at 1 µg/L, 10 µg/L, and 40 µg/L in drinking water for 6 consecutive months. Mice were sacrificed and lungs were obtained. (A) Lung tissue expression of occludin was measured by immunofluorescent staining. (B) Tissue expression of E-cadherin, SP-C, occludin, ZO-1, TGF-β and vimentin was measured by western blotting. Representative gel electrophoresis bands were shown (left panel). The expression levels of proteins were quantified by densitometry and normalized to the expression of β-actin (right panel) (n = 5 per group). Densitometry data are shown as mean ± SD. *P < 0.05 vs. control.

Fig. 3. Alveolar type II epithelial cells (ATII cells) were cultured in the presence of MC-LR at 0, 0.5, 5, 50 and 500 nM for 12 h unless otherwise indicated. (A) Cell-associated MC-LR was determined via western blotting. (B) Intracellular MC-LR was determined via immunofluorescent confocal microscopy ($\times 600$) following treatment with or without 500 nM MC-LR (C) Cell viability was measured using a dehydrogenase measurement-based assay. Six repeats were performed. * $P < 0.05$ vs. control. (D) Cell apoptosis was determined by FDA and PI staining ($\times 100$).

Fig 4.

Alveolar type II epithelial cells (ATII cells) were treated with MC-LR at 0, 0.5, 5, 50 or 500 nM for 48 h. (A) The expression of occludin was measured by immunofluorescent microscopy ($\times 600$). (B) The expression of CK18, CK19 and SP-C in ATII cells was measured by western blotting. Representative gel electrophoresis bands are shown (left panel). The expression levels of proteins were quantified by densitometry and normalized to the expression of β -actin (right panel). Three repeats were performed. Densitometry data are shown as means $\pm$ SD. * $P < 0.05$ vs. control. (C) The expression of E-cadherin, ZO-1 and vimentin in ATII cells was measured by western blotting. Representative gel electrophoresis bands are shown (left panel), and the expression levels of proteins were quantified by densitometry and normalized to the expression of β -actin (right panel). Three repeats were performed. Densitometry data are shown as means $\pm$ SD. * $P < 0.05$ vs. control.

Fig 5.

(A) Alveolar type II epithelial cells (ATII cells) were plated at confluency on filter inserts and cultured in 0, 0.5, 5, 50 or 500 nM of MC-LR for 12, 24, and 36 h. At the indicated time points, the transepithelial electrical resistance was measured. Three repeats were performed. * $P < 0.05$ vs. control. (B) Alveolar type II epithelial cells (ATII cells) were treated with MC-LR at 0, 0.5, 5, 50 or 500 nM for 12 h. The expression of p-ERK, ERK, P-MEK, MEK, p-AKT, AKT and PTEN in ATII cells was measured by western blotting. Representative gel electrophoresis bands are shown (left panel). The expression levels of proteins were quantified by densitometry and normalized to the expression of β -actin (right panel). Three repeats were performed. Densitometry data are shown as means $\pm$ SD. * $P < 0.05$ vs. control.

Table 1

Primers used for PCR analysis.

	Forward primer	Reverse primer
IL-1 β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
TNF- α	CTGAACTTCGGGGTGATCGG	GGCTTGTCACCTCGAATTTGAGA
GAPDH	AACCTTGGCATTGTGGAAGG	ACACATTGGGGTAGGAACA